Remarks

Claims 1, 3-16, and 18 are herein amended and new claims 19 and 20 are added. Support for amendment to claims 1 and 12 is found in claims 1, 8, and 12 as originally filed and p. 3 paragraph [005] of the specification as originally filed. Support for amendment to claims 3-11, 13-16, and 18 is found in these claims as originally filed respectively and the specification as filed.

Support for new claim 19 is found in claims 2 and 13 as originally filed. Support for new claim 20 is found in claim 8 as originally filed and on p. 8 paragraph [035] of the specification as originally filed.

No new matter has been added by the present amendment, and no new material presented that would necessitate an additional search on the part of the Examiner.

Applicants note with appreciation that the outstanding Office action withdraws rejection of claims 1, 3-7, and 10 under 35 U.S.C. §102(b) in view of Shimazaki et al. (U.S. patent number 5,957,038); and withdraws rejection of claims 1, 3-7, 10, 12, 15, and 16 under 35 U.S.C. §102(b) in view of Feygin et al. (U.S. patent number 6,315,957).

Upon entry of this Amendment and Response, claims 1, 3-16, and 18-20 are pending.

As a preliminary matter, Applicants believe that a brief description of independent claims 1 and 12 as here amended would be helpful, prior to characterizing each reference cited in the Office action and comparing the claims to references.

Claim 1 as here amended is directed to an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom for receiving and retaining the objects in which the objects are at least one object selected from the group of affinity beads and one or more living organisms, in which the receptacle is permeable on the bottom and on a portion of at least one side to permit the liquid to flow through the receptacle to intermix with the objects when the receptacle is inserted into a vessel containing the liquid, with the proviso that a portion of at least one side is not permeable.

Claims 12 as here amended is directed to an apparatus for intermixing objects with a liquid comprising a plurality of receptacles, each receptacle having sides and a bottom for containing the objects, in which the objects are at least one object selected from the group of affinity beads and one or more living organisms, each of the receptacles is permeable on the

bottom and on a portion of at least one side to permit the flow of the liquid therethrough, with the proviso that a portion of at least one side is not permeable, in which a vessel containing the solution, and means for repeatedly inserting the receptacles into the liquid in the vessel and then withdrawing the receptacle from the liquid to cause the liquid to first flow into the receptacle through the permeable portion when the receptacle is inserted into the liquid and then to flow outwardly from the receptacle through the permeable portion when the receptacle is withdrawn from the liquid.

Claims as here amended are novel

The Office Action on p. 3 ¶2 rejects claims 1, 3-16, and 18 under 35 U.S.C. §102(e) in light of Frondoza et al. (U.S. patent application number 2005/0147959, published July 7, 2005 and filed March 25, 2002). Applicants respectfully traverse.

The Office Action on pp. 3-4 ¶3 rejects claims 1, 3-7, 10-16, and 18 under 35 U.S.C. §102(e) in light of Feygin et al. (U.S. patent number 6,315,957, issued November 13, 2001). Applicants respectfully traverse.

According to criteria established in the Manual of Patent Examining Procedure, "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Manual of Patent Examining Procedure* § 2131 (M.P.E.P. 8th ed., Rev. 4, Oct. 2005), citing *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q. 2d 1051, 1053 (Fed. Cir. 1987). [emphasis added] Thus, the standard for rejection under 35 U.S.C. § 102 is identity.

Applicants show below that the subject matter of the present claims is not the same as the subject matter of each of the references cited.

Frondoza et al. (U.S. patent application number 2005/0147959, published July 7, 2005 and filed March 25, 2002)

Frondoza shows a two-component multi-well culture plate system (Frondoza et al. paragraph [0038] and Fig. 1). Frondoza shows microcarriers, for example: biopolymers such as collagen, gelatin, chitin, chitosan or chitosan derivatives, or fibrin; or particles of tissues such as bone or demineralized bone, cartilage, tendon, ligament, fascia, intestinal mucosa or other connective tissues. (Ibid. paragraph [0083]).

Frondonza's microtiter plate is fitted with an insert component having molded wells that fit into each well of the multi-well culture plate (Ibid. paragraph [0051]). At the bottom of each extension is placed a porous screen that covers the bottom of the well of the multi-well culture plate (Ibid. paragraph [0051]). The walls of Frondoza's multi-well culture plate are not permeable, i.e., liquid does not flow through the walls of the multi-well culture plate (Ibid. column 4 paragraph [0051] and Figure 1).

In contrast to Frondoza, claims 1 and 12 as here amended are directed to an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit the liquid to flow through the receptacle.

Frondoza fails to show an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, which is the subject matter of claims 1 and 12 as here amended. In contrast to the subject matter of claims 1 and 12 as here amended, Frondoza merely shows a multi-well culture plate such that at the bottom of each extension is placed a porous screen that covers the bottom of the well of the multi-well culture plate (Ibid. paragraph [0051]). The walls of Frondoza's multi-well culture plate are not permeable, i.e., liquid does not flow through the walls of the multi-well culture plate (Ibid. column 4 paragraph [0051] and Figure 1).

For these reasons, Frondoza is not the same as claims 1 and 12 as here amended. Therefore these claims as here amended are novel under 35 U.S.C. §102(e). Claims 3-11, 13-16, and 18 depend directly or indirectly from claims 1 or 12 and incorporate the subject matter of claims 1 or 12 as here amended and contain additional subject matter, and therefore these claims also are novel in view of Frondoza.

Applicants respectfully request that rejection of claims 1 and 12 as here amended and claims 3-11, 13-16, and 18 under 35 U.S.C. §102(e) in view of Frondoza et al. be withdrawn.

Feygin et al. (U.S. patent number 6,315,957, issued November 13, 2001)

Feygin et al. shows an article for segregating solid support media from liquid (Feygin et al. Abstract). The article includes a filter pocket plate consisting of a plate with a plurality of

holes in which a mesh-like material projects through each hole in the plate (Ibid. column 1 lines 63-67, and Fig. 2). The pocket plate engages a vessel having wells suitable for retaining liquid (Ibid., column 2 line 4-10, and Fig. 3). Feygin's filter pocket plate is permeable throughout (Ibid. Fig. 2 and Fig. 3).

Feygin shows that this device is used for carrying-out solid-phase synthesis of chemical compounds (Ibid. column 5 line 54 to column 6 line 19). The purpose of Feygin's apparatus is for chemical operations (Ibid. Abstract), to link a reactive functionality to a solid support so the functionality can be modified and then cleaved (Ibid. Fig. 7). The liquid in Feygin includes chemical building blocks for reacting with the reactive functionality, so that the reaction produces a chemical compound (Ibid. column 2 lines 16-27).

Nowhere does Feygin show an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, with the proviso that a portion of at least one side is not permeable, which is the subject matter of claims 1 and 12 as here amended. In contrast to the subject matter of claims 1 and 12 as here amended, Feygin's filter pocket plate is permeable throughout (Ibid. Fig. 2 and Fig. 3).

For these reasons, claims 1 and 12 as here amended are novel in view of Feygin et al. under 35 U.S.C. §102(e). Claims 3-7, 10-11, 13-16, and 18 depend directly or indirectly from claims 1 or 12 and incorporate the subject matter of claims 1 or 12 as here amended and contain additional subject matter, and therefore these claims are also novel in view Feygin et al.

Applicants respectfully request that rejection of claims 1 and 12 as here amended and claims 3-7, 10-11, 13-16, and 18 under 35 U.S.C. §102(e) in view of Feygin et al. be withdrawn.

Claims as here amended are not obvious

The Office Action on pp. 5-6 ¶4 rejects claims 1, 3-7, 10-16, and 18 under 35 U.S.C. §103(a) in light of Feygin et al. in combination with Reeve et al. (International patent application number WO 91/12079, published August 22, 1991) and/or Valkirs et al. (U.S. patent number 6,348,318, issued February 19, 2002). Applicants respectfully traverse.

As a preliminary matter, the Office action states with respect to the present rejection of claims 1, 3-7, 10-16, and 18 under 35 U.S.C. §103(a) that "Feygin, however, not expressly state

that the liquid solution is a lysate that contains proteins that become bound to the affinity beads when the solution and the affinity beads are intermixed." See Office action p. 5 ¶4.

Claims 1 and 12 as amended are directed to an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom for receiving and retaining the objects in which the objects are at least one object selected from the group of affinity beads and one or more living organisms, in which the receptacle is permeable on the bottom and on a portion of at least one side to permit the liquid to flow through the receptacle to intermix with the objects when the receptacle is inserted into a vessel containing the liquid, with the proviso that a portion of at least one side is not permeable.

Applicants show below that the combination of Feygin, Reeve, and Valkirs does not render obvious claims 1 and 12 as here amended. The Supreme Court in *Graham v. John Deere*, 383 U.S. 1 provided an analytical construct to be used when determining whether claims are obvious under 35 U.S.C. §103(a) in view of prior art. One aspect of this analytical construct includes characterizing the prior art, as a background for a legal analysis.

Feygin et al. (U.S. patent number 6,315,957, issued November 13, 2001)

Feygin is characterized above.

Feygin et al. teaches an article for segregating solid support media from liquid (Feygin et al. Abstract). The article includes a filter pocket plate consisting of a plate with a plurality of holes in which a mesh-like material projects through each hole in the plate (Ibid. column 1 lines 63-67, and Fig. 2). In contrast to the subject matter of claims 1 and 12 as here amended, Feygin's filter pocket plate is permeable throughout (Ibid. Fig. 2 and Fig. 3).

Nowhere does Feygin teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, with the proviso that a portion of at least one side is not permeable, which is the subject matter of claims 1 and 12 as here amended.

Thus Feygin alone does not render obvious the subject matter of claims 1 and 12 as here amended. Applicants show below that Reeve, alone or in combination, does not cure the defects of Feygin.

Reeve et al. (Internation patent application number WO 91/12079, published August 22, 1991)

Reeve et al. shows methods of isolating macromolecules using magnetically attractable beads. In fact, Reeve et al. is titled, "[m]ethod to isolate macromolecules using magnetically attractable beads which do not specifically bind the macromolecules." [emphasis added].

Reeve shows a method of treating a solution of a polymer by the use of magnetically attractable beads, the method includes the steps of: suspending magnetically attractable beads in a solution; and applying a magnetic field to draw down a precipitate of the beads and the associated polymer (Reeve, p. 4 lines 6-20). In fact, Reeve's states, "[t]he key to the invention is the use of magnetically attractable beads ..." (Ibid., p. 4 lines 21-23; emphases added). Reeve shows only separation methods based on the use of magnetic beads and application of a magnetic field (Ibid. p. 6-16).

Reeve's further states:

> It is a feature of the invention that the magnetic beads do not specifically bind the polymer. By this feature, the present invention is distinguished from many prior techniques which involve providing a coating on the surface of magnetic beads designed to specifically bind the substance to be drawn down out of solution [Ibid. p. 5 line 32 to p. 6 line 2; emphases added].

The separation method used in Reeve is based on an entirely different technology using different physical principles than the apparatus of the present claims. Factual analysis shows that the separation method used in Reeve requires magnetically attractable beads and application of a magnetic field. In contrast, in the apparatus of the present claims for intermixing objects and a liquid, the objects are retained in the receptacle and the liquid flows through the permeable portions of the receptacle, which is a completely different apparatus than what is shown in Reeve.

Reeve does not teach or suggest any receptacle that is permeable of any type. Reeve does not teach or suggest a vessel to which is inserted the receptacle that is permeable.

Most important, Reeve fails to teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, with the proviso that a portion of at least one side is not permeable, which is the subject matter of claims 1 and 12 as here amended.

Therefore, Reeve does not cure the defects of Feygin, alone or in combination, and fails to render obvious the subject matter of claims 1 and 12 as here amended. Applicants show below that Valkirs, alone or in combination, does not cure the defects of Feygin and Reeve in combination.

Valkirs et al. (U.S. patent number 6,348,318, issued February 19, 2002)

Valkirs shows a method that uses magnetic particles to concentrate target analytes (Valkirs, Abstract, and Fig. 1). Valkirs is titled, "[m]ethods for concentrating ligands using magnetic particles". (emphasis added) Valkirs shows types of magnetic beads, for example, iron oxide particles, and commercial suppliers of magnetic beads (Ibid. column 5 lines 14-48).

The Valkirs' method involves adding a magnetic bead having attached a capture moiety to a sample that has a target analyte binding moiety to form a magnetic bead-bound target complex (Ibid. column 1 lines 54-61). A magnetic field is applied to the sample to collect the magnetic bead-bound target complex (Ibid. column 1 lines 61-66).

Separation of Valkirs' target molecules requires a magnetic field to concentrate the sample at the bottom of a container. The liquid is then aspirated or poured from the container (Ibid. column 12 lines 1-14).

The separation method used in Valkirs is based on an entirely different technology using different physical principles than the apparatus of the present claims. Factual analysis shows that the separation method used in Valkirs requires magnetically attractable beads and application of a magnetic field. In contrast, in the apparatus of the present claims for intermixing objects and a liquid, the objects are retained in the receptacle and the liquid flows through the permeable portions of the receptacle, which is a completely different apparatus than what is shown in Valkirs.

Valkirs does not teach or suggest any receptacle that is permeable of any type. Valkirs does not teach or suggest a vessel to which is inserted the receptacle that is permeable.

Most important, Valkirs fails to teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, with the proviso that a portion of at least one side is not permeable, which is the subject matter of claims 1 and 12 as here amended.

Therefore, Valkirs does not cure the defects of Feygin and Reeve, alone or in combination, and fails to render obvious the subject matter of claims 1 and 12 as here amended.

Claims 3-7, 10-11, 13-16, and 18 depend directly or indirectly from claims 1 or 12 and incorporate the subject matter of claims 1 or 12 as here amended and contain additional subject matter, and therefore these claims also are not obvious in view of the combination of Feygin, Reeve, and Valkirs.

Legal analysis of references combined

According to a summary of criteria in the *Manual of Patent Examining Procedure,* "[t]o establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure." [emphases added] M.P.E.P. §2142 (8th Ed. Rev.2, May 2, 2004); *In re Vaeck,* 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

A recent decision by the U.S. Supreme Court, *KSR International Co. v. Teleflex Inc.* 550 U.S.____ (2007), discusses criteria for showing a motivation to combine numerous prior art references in a determination that a claimed invention is obvious. The U.S. Supreme Court in *KSR* explained that "[t]here is no necessary inconsistency between the idea underlying the TSM [teaching, success, motivation] test and the *Graham* analysis." *KSR International Co.* 550 U.S.____ at p. 15. In fact, the court explains "... it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the newly claimed invention does." Id.

Applicants respectfully traverse the above rejection, and show that the facts of the case and the relevant case law indicate that the invention would not have been obvious to one of ordinary skill in the art at the time the application was filed because the underlying facts show that the criteria for a *prima facie* rejection have not been met.

Failure of the cited prior art to teach or suggest all the claim limitations

To establish a *prima facie* case for obviousness of a claimed invention, all of the claim limitations must be taught or suggested by the prior art. M.P.E.P. §2143.03; *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974).

An element of claims 1 and 12 as here amended is an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, with the proviso that a portion of at least one side is not permeable.

Feygin merely teaches and suggests a filter pocket plate that is basically a sieve, and is permeable throughout (Ibid. Fig. 2 and Fig. 3). Reeve and Valkirs teach and suggest only a non-permeable vessel. In fact, nothing in Reeve and Valkirs teaches or suggests any receptacle that is permeable of any type.

Thus the combination of Feygin, Reeve, and Valkirs fail to render obvious the subject matter of claims 1 and 12 as here amended because the prior art references when combined do not teach or suggest all the claim limitations, as required by M.P.E.P. §2142. Therefore a *prima facie* case of obviousness of claims 1 and 12 as here amended has not been made.

Claims 3-7, 10-11, 13-16, and 18 that depend directly or indirectly from claims 1 or 12 and incorporate all of the subject matter of claims 1 or 12 as here amended and contain additional subject matter also are not obvious in light of the cited references.

Therefore Applicants respectfully request withdrawal of rejection of claims 1 and 12 as here amended and claims 3-7, 10-11, 13-16, and 18 under 35 U.S.C. §103(a).

Claims as here amended are definite

The Office action on p. 2 ¶1 rejects claims 1 and 12 under 35 U.S.C. §112 ¶2 alleging that the term "small" as it relates to the size of the objects in claims 1 and 12 is indefinite. Applicants respectfully traverse.

As a preliminary matter, claims 1 and 12 are here amended, and as amended are directed inter alia to at least one object selected from the group of affinity beads and one or more living organisms.

One of ordinary skill in the art of biochemistry, reading the specification at the time it was filed, would have understood the size of the objects in claims 1 and 12 as here amended, as shown below.

Claims 1 and 12 as here amended are directed to objects selected from the group of affinity beads and one or more living organisms. The specification as filed on p. 7 paragraph [024] shows an example of a type of affinity bead, viz., TALON beads (commercially available from Biosciences Clontech). One of ordinary skill in the art of biochemistry would have understood the size of these commercially available affinity beads at the time the application was filed because the commercial literature is replete with this information. See Appendix **A** attached hereto which is an excerpt from the BD Biosciences Clontech catalog describing TALON affinity beads. This catalog shows exemplary affinity bead size ranges, such as, 45μm to165μm, 60μm to 160μm, and 300μm to 500μm. See Appendix A, p. 13.

Further, the specification as filed on p. 8 paragraph [035] provides examples of living organisms, embryos and larvae. One of ordinary skill in the art of biochemistry would have been well informed regarding sizes of living organisms of choice, at the time the application was filed because the scientific literature is replete with this information. See Appendix **B** attached hereto, Dong et al. (Crop Science 43(3):1068-1071, 2003). This article shows exemplary sizes of embryos, viz., widths that range from about 300μm to about 560μm and lengths that range from about 1300μm to about 1900μm. See Appendix **B**, enlarged view of Fig. 1 on last page of article.

In fact, the specification as filed on p. 5 paragraphs [018] to [019] shows exemplary pore sizes for the permeable portion of the receptacle, for example, 25μm, 30μm, 33μm, and 41μm. One of ordinary skill in the art of biochemistry would have understood, reading the application at the time it was filed, to choose a pore size that is smaller than the size of the objects so that the receptacle is capable of receiving and retaining the objects of claims 1 and 12 as here amended.

For these reasons, one of ordinary skill in the art in biology or biochemistry, reading the specification at the time the application was filed, would clearly have understood the size of the objects that are the subject matter of claims 1 and 12 as here amended.

Therefore rejection of claims 1 and 12 as here amended under 35 U.S.C. §112 ¶2 can be withdrawn, an action that is respectfully requested.

The Office action on p. 2 ¶1 rejects claims 8 and 9 under 35 U.S.C. §112 ¶2.

Claim 1 as here amended is directed to apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom for receiving and retaining the objects, in which the objects are at least one object selected from the group of affinity beads and one or more living organisms.

Therefore rejection of claims 8 and 9 under 35 U.S.C. §112 ¶2 can be withdrawn, an action that is respectfully requested.

Summary

On the basis of the foregoing amendments and reasons, Applicants respectfully submit that the pending claims are in condition for allowance, which is respectfully requested. If there are any questions regarding these remarks, the Examiners are invited and encouraged to contact Applicants' representatives at the telephone number provided.

Respectfully submitted,

Adam Schoen

Adam M. Schoen, Reg.Number 58,576
Sonia K. Guterman, Reg.Number 44,729
Attorneys for Applicants
Lawson & Weitzen, LLP
88 Black Falcon Ave., Suite 345
Boston MA 02210-2481
Tel: (617) 439-4990
Fax: (617) 439-3987
Customer Number 48425

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Appendix A

TALON™ Products

For polyhistidine-tagged protein purification

Product	Application
TALON™ Metal Affinity Resin Resin ready for loading in columns for small or medium-scale purification of His-tagged proteins. Purify > 5 mg protein using 1 ml of resin.	For purification of most cytosolic and secreted His-tagged proteins by small-scale or batch/gravity flow, under native or denaturing conditions.
TALON™ Superflow Resin Specially designed for quick and effective purification of His-tagged proteins at high flowrates and medium-pressure (up to 150 psi).	For FPLC, medium-pressure chromatography, or scale-up for production applications.
TALON™ CellThru Novel IMAC resin designed for quick purification of His-tagged proteins by direct capture.	For small-scale single-use applications such as verifying positive transformants for His-tagged protein expression levels, or trial-level purification protocols.
TALONspin™ Columns Ready-made spin columns containing TALON-NX™ resin for the simultaneous purification of several His-tagged proteins in parallel in only 30 minutes.	For small-scale single-use applications such as verifying positive transformants for His-tagged protein expression levels, or trial-level purification protocols.
TALON™ Purification Kit Convenient kit containing TALON resin, columns, and all the buffers necessary to extract, wash, and elute His-tagged proteins. This kit provides the ideal place to start when using TALON in your applications.	
TALON™ Disposable Columns Two different types of disposable columns—one for use with TALON Resin for regular His-tagged protein purification and one for use with CellThru Resin for purification from crude lysates.	
TALON™ Buffer Kit Supplemental kit containing concentrated forms of optimized buffers for extracting, washing, and eluting proteins.	

TALON™ Metal Affinity Resin

TALON Resins are durable, cobalt-based IMAC resins designed to purify recombinant poly-histidine-tagged proteins (Bush *et al.*, 1991). These resins are compatible with many commonly used reagents, and allow protein purification under native and denaturing conditions. They can be used with all prokaryotic and eukaryotic expression systems in a variety of formats, including small- (mini-) scale batch screening, large-scale batch preparations, and methods using gravity-flow columns and spin columns.

Introduction

Proteins have evolved very complex structures in order to perform a diverse array of functions. As a result, their physicochemical properties vary greatly, posing difficulties when developing versatile purification protocols. A host of purification methods have been developed that capitalize on the general physical properties of proteins. One of the quickest and easiest ways to purifying a protein is to use affinity chromatography since it is generally a more selective method of purification, which lets the protein of interest can be purified in one or two steps. However, many proteins have not been characterized sufficiently, or do not have any known strong binding properties that can be utilized for purification. One way to circumvent this problem is to incorporate a purification tag into the primary amino acid sequence of a target protein, thus constructing a recombinant protein with a binding site that allows purification under well-defined, generic conditions.

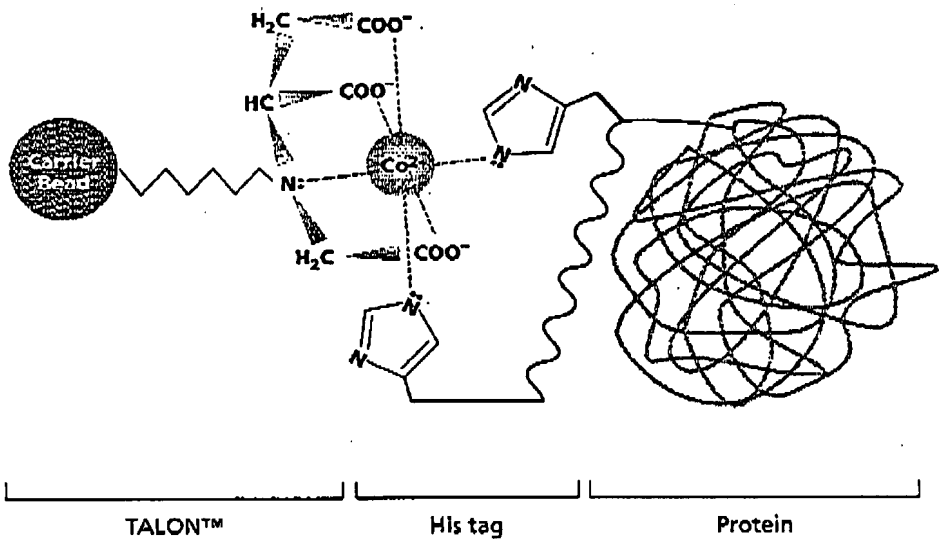

Figure 2. Molecular mechanism of histidine binding to TALON™ Resin.

TALON™ Metal Affinity Resin...*cont.*

IMAC technology

TALON Resin is an immobilized metal affinity chromatography (IMAC) resin based on our innovative, patented technology. IMAC was introduced in 1975 as a group-specific affinity technique for separating proteins (Porath *et al.*, 1975). This principle is based on the reversible interaction between various amino acid side chains and immobilized metal ions. Depending on the immobilized metal ion, different side chains can be involved in the adsorption process. Most notably, histidine, cysteine, and tryptophan side chains have been implicated in protein binding to immobilized transition metal ions and zinc (Porath, 1985; Sulkowski, 1985; Hemdan & Porath, 1985a; Hemdan & Porath, 1985b; Zhao *et al.*, 1991).

His-tag purification

Histidines exhibit highly selective binding to certain metals and have great utility in IMAC. Under conditions of physiological pH, histidine binds by sharing electron density of the imidazole nitrogen with the electron-deficient orbitals of transition metals. Although only three histidines may bind transition metals under certain conditions, six histidines reliably bind transition metals in the presence of strong denaturants such as guanidinium (Hochuli *et al.*, 1987). Such protein tags are commonly referred to as 6xHis tags. We have developed several other His-tag purification systems, including 6xHN and HAT. These tags possess characteristics favorable for binding to IMAC resins and improve protein solubility and yield (see page 22).

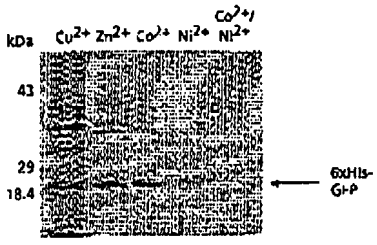

Figure 3. The cobalt ion has higher affinity and specificity for His-tagged proteins. The indicated metals were immobilized onto sepharose CL-6B (Pharmacia) using TALON's unique tetradentate chelator. 20 µl of eluate from the indicated resin was electrophoresed on a 12% polyacryl-amide gel and stained with Coomassie blue.

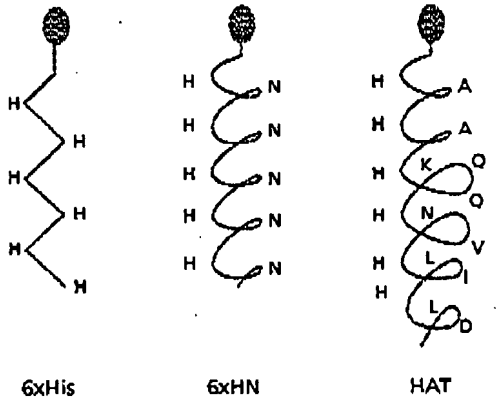

Figure 4. Hypothetical structures of commonly used histidine-affinity tags.

Unique Properties of TALON™ Resin

Reactive core contains cobalt

TALON has a remarkable affinity and specificity for His-tagged proteins (Figures 3, 7, 9, 10, & 11). The TALON reactive core, which contains cobalt, has strict requirements for the spatial positioning of histidines. Only adjacent histidines or specially positioned, neighboring histidines are able to bind cobalt in this reactive core. In nickel-based resins (i.e. Ni-NTA Resin), these spatial requirements are less strict. Therefore, nickel-based resins are also able to bind histidines located in places other than the protein's His-tag (Figure 3).

Uniform matrix

Cobalt-based resins have a more uniform structure than nickel-based resins. All reactive sites in TALON resin look like three-dimensional pockets, similar to the one drawn in Figure 2. In these pockets, cobalt is bound to three carboxyl groups and one nitrogen atom, and is able to bind to two other ligands, i.e. two histidines. In this configuration, cobalt is bound very tightly and does not leak out of the resin. Nickel-based resins are less homogeneous in structure because nickel ions can form two different coordination structures. One of them is a three-dimensional pocket, similar to TALON. The other structure is planar (flat). In this distorted, planar structure nickel is bound to only two carboxyl groups and one nitrogen atom. Since this binding is not very strong, planar reactive cores are not able to hold nickel ions very tightly. This leads to leaching of the nickel ion from the resin.

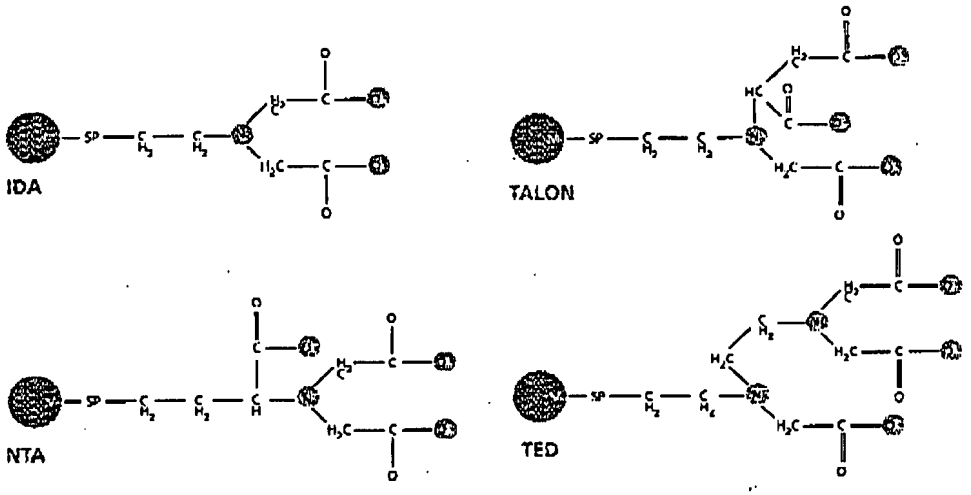

Figure 5. Chemical structures of chelating ligands used in IMAC. Binding groups are colored purple. SP = spacer, M = matrix.

Unique Properties of TALON™ Resin...*cont.*

Comparison with Ni-NTA resin

TALON exhibits subtle yet important differences in character when compared with nickel IMAC resins. For example, nickel-based IMAC resins often exhibit an undesirable tendency to bind unwanted host proteins containing exposed histidine residues (Kashor *et al.*, 1993). In contrast, TALON binds polyhistidine-tagged proteins with enhanced selectivity over nickel-based resins, and it also exhibits a significantly reduced affinity for host proteins (see page 6; Sulkowski, 1989). This characteristic offers two practical advantages. First, virtually no background proteins are bound to TALON when the sample is applied; consequently, cumbersome washing procedures are not generally required before protein elution. Second, polyhistidine-tagged proteins elute from TALON under slightly less stringent conditions—a slightly higher pH or lower imidazole concentration—than with nickel IMAC resins. Elution occurs when the imidazole nitrogen (pKa of 5.97) is protonated, generating a positively charged ammonium ion, which is repelled by the positively charged metal atom. Alternatively, simply adding imidazole to the elution buffer can competitively elute the bound polyhistidine-tagged protein because imidazole is structurally identical to the histidine side chain and therefore out-competes histidines for resin binding.

Why metal leaching is detrimental to protein purification

During protein purification, metal separates from the reactive core of the purification resin and flows down the column. This is called metal leaching. When metal leaching occurs, it reduces the number of reactive sites available for protein binding on the column, therefore reducing the amount of purified protein obtained.

All metals will leach out of a resin, but nickel leaches more readily than cobalt. Nickel can also precipitate proteins by forming salt bridges, can be toxic to cells and tissues, and can damage purified protein because of its nucleophilic properties. For these reasons, TALON Resin employs cobalt in its reactive core rather than nickel.

Table I. Comparison of TALON™ Metal Affinity Resin vs. Ni-NTA Resin

	TALON™ Resin	Ni-NTA Resin
Metal	Cobalt	Nickel
Metal Ion Complex	Strong	Weak, metal leakage results in lower yields of His-tagged protein and contamination by nonspecific proteins
Sensitivity to β-mercapto-ethanol	Low to negligible sensitivity when concentration < 30 mM	High, resulting in low yields of His-tagged protein
Performance under denaturing conditions	++	+
Performance under nondenaturing conditions	+	+
Reusability	++	+
Nonspecific protein binding	None	Significant

Protein Purification with TALON™

Denaturing vs. native conditions

Purification conditions

Deciding whether to use native or denaturing purification conditions depends on protein location, solubility, accessibility of the histidine tag, downstream applications, and preservation of biological activity. TALON Resin retains its protein binding specificity and yield in a variety of purification conditions. It is stable in both denaturing and native (nondenaturing) conditions.

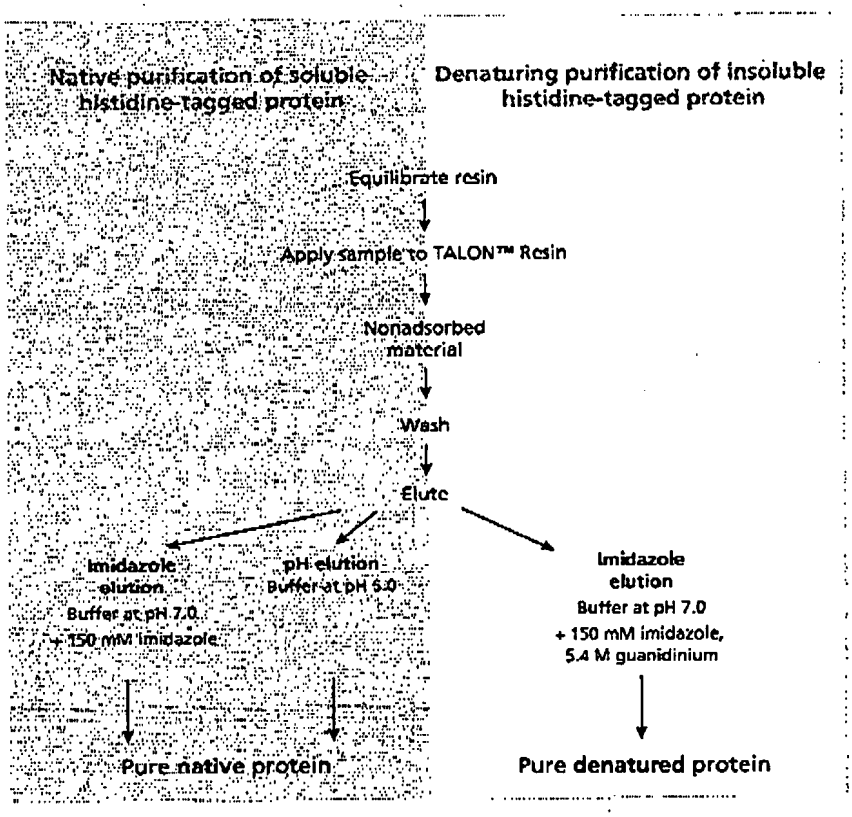

Figure 6. Native vs. denaturing purification procedures.

Protein Purification with TALON™...*cont.*

Why use denaturing conditions?

Denaturants, such as 6 M guanidinium, enhance protein solubility. Because proteins that are overexpressed in prokaryotic systems sometimes form insoluble aggregates called inclusion bodies, you may need to purify proteins under denaturing conditions. Strong denaturants such as 6 M guanidinium or 8 M urea completely solubilize inclusion bodies and 6xHis-tagged proteins. Under denaturing conditions, the 6xHis tag on a protein will be fully exposed so that biding to the matrix will improve, and the potential for nonspecific binding will be greatly reduced.

6xHis-tagged proteins purified under denaturing conditions can be used directly in subsequent applications, or may need to be renatured and refolded. Protein renaturation and refolding can be performed prior to elution from the column (Holzinger *et al.*, 1996) or in solution (Wingfield *et al.*, 1995). However, yields of recombinant proteins will be lower than under native conditions. This is because urea and guanidinium molecules compete with histidines for binding to metal.

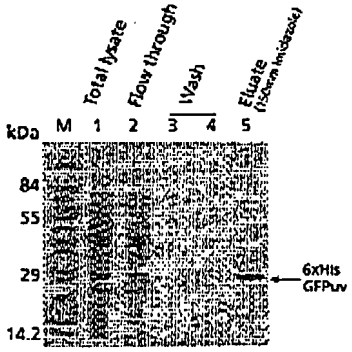

Figure 7. Purification of 6xHis-GFPuv under denaturing conditions. The fusion protein was purified in 8-M urea using TALON resin. M=molecular weight markers.

Protein Solubility

Protein solubility is largely dependent on two factors: the hydrophobicity of the amino acids in the polypeptide backbone, and the ability of the protein to fold correctly. Researchers can use a number of standard methods developed to influence protein solubility. At the level of protein expression, protein solubility can be changed by changing the level of expression. In *E. coli*, Recombinant proteins that are overexpressed are frequently found to form protein aggregates called inclusion bodies. Such structures are believed to be masses of the expressed protein that have not folded correctly. Depending on your application, inclusion body formation can frequently be overcome by either reducing the level of expression. Alternatively, switching from 6xHis tag to HAT tag may help to increase protein solubility (see page 22 for details). Sometimes switching to a eukaryotic expression system helps the solubility of expressed protein because eukaryotes have the ability to add post-translational modifications or utilize chaperone-assisted protein folding. At the level of protein purification, solubility can be increased by changing the temperature or salt concentration, or using reducing agents and denaturants in the method.

Protein Purification with TALON™...*cont.*

Why use native conditions?

Purifying a protein under native conditions is the most efficient method of retaining its biological activity. In order to use native conditions the protein must be soluble. Purification of proteins under native conditions is advantageous not only because you avoid the renaturation step at the end of the purification, but also because native purification will usually copurify enzyme subunits, cofactors, and associated proteins present in the cells (Le Grice, *et al.*, 1990; Flachmann & Khulbrandt, 1996). When renaturing protein after a denaturing purification, it is uncommon to regain more than 2–5% of the activity.

One disadvantage of using native conditions is that unrelated, nontagged proteins are more likely to be nonspecifically bound to the TALON Resin than with denaturing conditions. However, the nonspecific binding can be reduced by including a low concentration of imidazole (5–20 mM) in the wash buffer.

Sometimes the 6xHis tag is concealed by the tertiary structure of the soluble protein, so the protein must be denatured before it can be purified. If purification can only be performed under denaturing conditions, and this does not suit the downstream applications, an inaccessible tag can be moved to the other terminus of the protein. Alternatively, a larger tag like HAT or 6xHN can be used (see page 22).

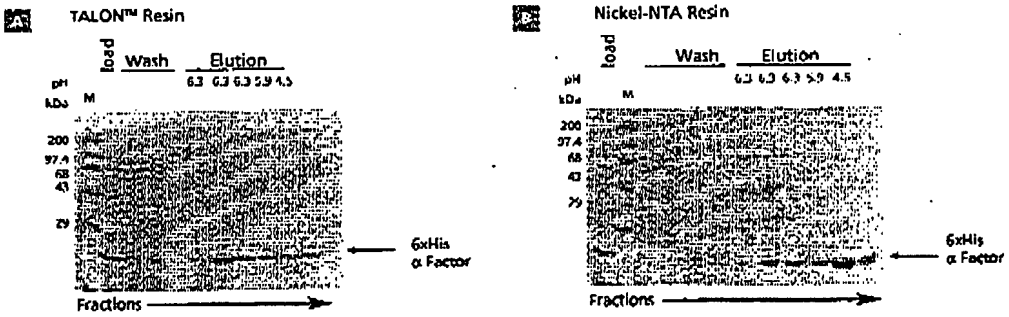

Figure 8. Purification of 6xHis proteins under native conditions compared to purification using Ni-NTA. In comparison with Ni-NTA resin, TALON is more specific for His-tagged proteins. His-tagged proteins can be eluted from TALON at more neutral conditions (pH = 6.3) than from Ni-NTA resins (pH = 4.5). 6xHis-tagged prepro-α-factor was expressed in *E. coli*, lysed and loaded onto each gravity flow column and eluted by a step-wise pH gradient. Purified fractions were analyzed by SDS-PAGE. M=molecular weight markers.

Protein Purification with TALON™...*cont.*

TALON resin preserves the native activity of purified proteins. Figure 9 shows that biological activity of green fluorescent protein (GFPuv) is preserved when purified using TALON Superflow Resin.

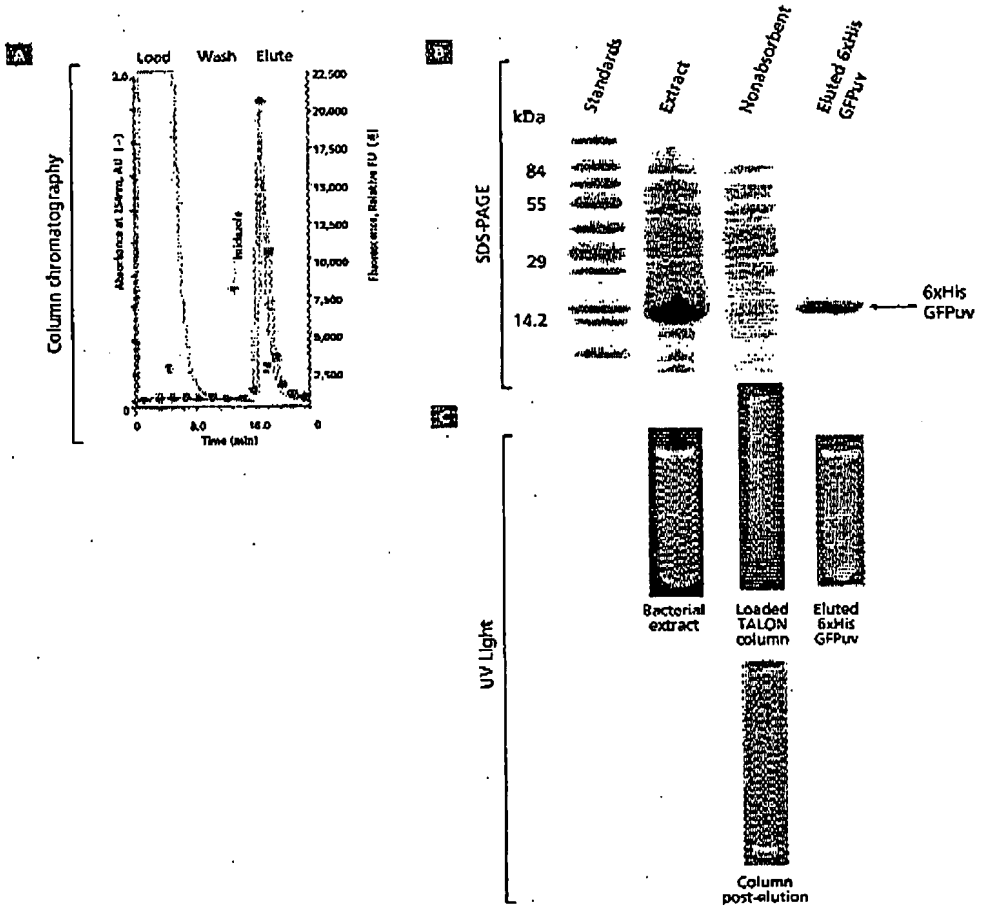

Figure 9. Native purification with TALON™ preserves biological activity of proteins. Fresh cells (0.5 g) expressing 6xHis-GFPuv were extracted in 5 ml of 50 mM sodium phosphate; 0.3 M NaCl, pH 7.0 **Panel A.** Elution profile of GFP which was loaded, washed with the same buffer, and eluted with a step gradient of imidazole (150 mM). **Panel B.** Fractions were analyzed by SDS-PAGE. **Panel C.** Active, intact GFP protein visualized under UV light.

Purification with β-Mercaptoethanol

Why use β-mercaptoethanol in protein purification?

Some intracellular proteins contain reduced sulfhydryl (-SH) groups that are important for the biological activity and structure of the protein. Adding β-mercaptoethanol helps to preserve those -SH groups during purification.

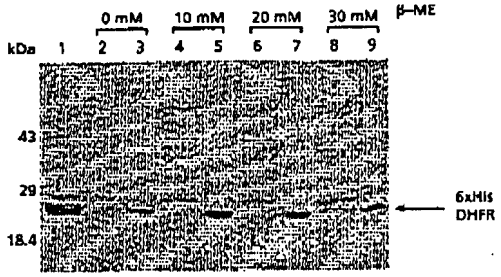

Figure 10. Native Purification of 6xHis protein in the presence of β-mercaptoethanol. N-terminal 6xHis-tagged mouse DHFR (19.5 kDa) was expressed in *E. coli*. 2 ml of lysate was purified using gravity flow on TALON resin in increasing concentrations of β-mercaptoethanol. Even lanes: 20 µl of non-adsorbed material. Odd lanes: 5 µl of eluate.

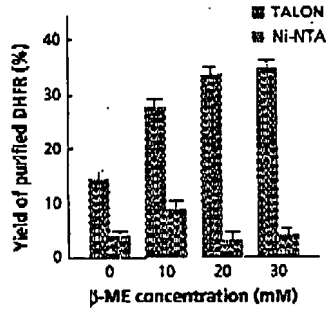

Figure 11. Yields of purification in the presence of β-mercaptoethanol compared to Ni-NTA resin. N-terminal 6xHis DHFR was expressed and purified under native conditions. Protein concentrations were determined by Bradford assay. Yields are expressed as a percentage of total protein in the cell lysate.

Formats of TALON™ Resin

Physicochemical properties

Table II. Physicochemical properties of TALON™ Resins

Features	TALON™ Superflow TALON™ Resin	TALON™ Resin	TALONspin™ CellThru	Columns
Batch/gravity flow applications	Yes	Yes	Yes	No
FPLC applications	No	Yes	Yes	No
Scale	Analytical, preparative, production	Analytical, preparative, production	Preparative, production	Analytical
Capacity (mg protein/ml adsorbent)	5–10	5–8	5–10	2–4
Matrix	Sepharose 6B-CL (6% cross-linked agarose)	Superflow (6% cross-linked agarose)	Uniflow (4% cross-linked agarose)	Silica
Bead size (µm)	45–165	60–160	300–500	16–24
Maximum linear flow rate (cm/hr)*	30	3,000	800	n/a
Maximum volumetric flow rate (ml/min)*	0.5	50	13	n/a
Recommended volumetric flow rate (ml/min)	0.3	1.0–5.0	1.0–5.0	0.3
Maximum pressure	2.8 psi 0.2 bar 0.02 MPa	150 psi 10 bar 0.97 MPa	9 psi 0.62 bar 0.02 MPa	n/a
pH stability (duration)	2–14 (2 hr) 3–14 (24 hr)	2–14 (2 hr) 3–14 (24 hr)	2–14 (2 hr) 3–14 (24 hr)	2–8.5 (2 hr) 2–7.5 (24 hr)
Protein exclusion limit (Da)	4×10^7	4×10^6	2×10^7	n/a

*For washing and elution only.

Protein Purification Procedures

Batch

In batch purification, the sample is applied to a tube containing resin. After incubation, the tube is centrifuged and the supernatant is discarded. The resin is washed with buffer and centrifuged. Then, elution buffer is added and the supernatant is collected after centrifugation.

Batch/gravity flow

Batch/gravity-flow purification means the protein is bound to the resin in solution and then the protein-resin mixture is applied to a column for washing and elution. This procedure gives efficient binding of 6xHis-tagged proteins, most notably when the 6xHis tag is not completely accessible or when the desired protein in the lysate is present in low concentration. By taking this approach, you optimize the time of contact between the resin and your sample. This method is also simpler and requires less equipment than other methods. Batch/gravity flow is usually intended for small-scale purification.

Standard column chromatography

In column purification, the protein binds the resin directly in the column, not in solution as with batch and gravity-flow purification. The resin is first packed into the column and equilibrated with lysis buffer. Then, the cell lysate is applied to the column. Washing and elution steps follow just as in the batch purification procedure. This method affords higher purity of the final product and is also faster than other methods.

FPLC (Fast Protein Liquid Chromatography)

FPLC is a protein purification technique utilizing inert materials, such as glass or plastic, to purify proteins without any metal leaching from the instruments into the protein sample. This method permits you to run chromatography purification at flow rates of 10 ml/min/cm^2 under medium pressure (up to 3 MPA). High flow rates are desirable because you obtain purified protein much more quickly. Fast purification limits the amount of time your protein spends in the presence of proteases (and other impurities) so you get a higher yield of purified product. However, in order to use such high flow rates, the resin must be able to withstand the associated pressure and maintain permeability. TALON Superflow Resin contains specially cross-linked agarose beads that are stronger than conventional agarose beads, so they can be used in FPLC applications. In addition, TALON Superflow beads have high permeability which results in decreased back pressure at elevated flowrates.

Spin column

Spin-column purification is intended for very small-scale, analytical-grade protein purification. This method is employed when purifying only small amounts of protein from many different samples.

Purification using FPLC

TALON™ Resin is available in the TALON™ Superflow format, which is useful for a variety of applications, including medium-pressure applications with FPLC systems at back pressures of up 150 psi (1 MPa). TALON Superflow can be used at high linear flow rates—up to 5 ml/min/cm^2. This resin is recommended if short purification times are essential, or if purification protocols developed for small or medium scale volumes scale need to be scaled up for larger volumes.

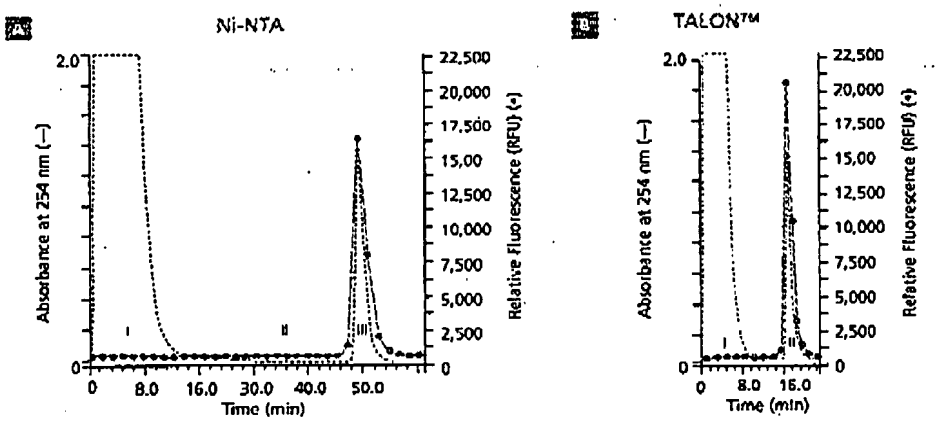

Figure 12. FPLC purification of 6xHis-GFPuv with TALON™ Superflow. Nickel-NTA (Panel A) requires longer washing and lower flow rates to purify 6xHis-GFPuv than TALON Superflow (Panel B). Protein was extracted in 50 mM sodium phosphate, 0.3 M NaCl, pH 7.0. Panel A. 3.2 ml culture filtrate was loaded at 0.5 ml/min. Then nonadsorbed material was washed in the same buffer with 10 mM imidazole. Protein was eluted with 20 mM imidazole (peak II) and 250 mM imidazole (peak III). Panel B. 3.2 ml culture filtrate was loaded at 1 ml/min. Then, nonadsorbed material was washed with the same extraction buffer and eluted with 150 mM imidazole (peak II).

Purification using FPLC...*cont.*

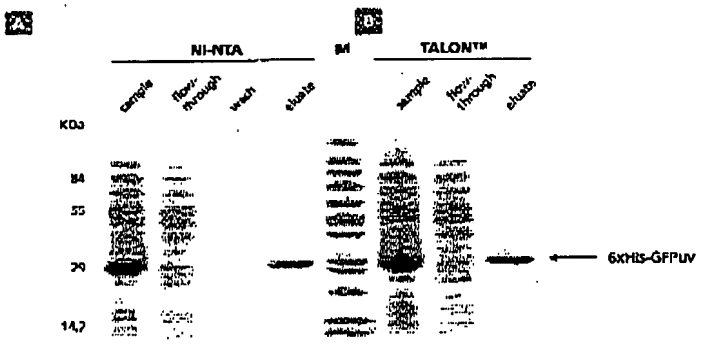

Figure 13. SDS-PAGE of FPLC fractions from 6xHis-GFPuv purification. FPLC purification fractions from the peaks in Figure 11. Purification with TALON Superflow requires less washing with exceptional results.

Table III. TALON®-compatible reagents

Reagent	Acceptable concentration
β-Mercaptoethanol	10 mM (with caution)
CHAPS	1% (with caution)
Ethanol	30% (only for storage)
HEPES	50 mM
Glycerol	20%
Guanidinium-HCl	6 M
Imidazole	200 mM at pH 7.0–8.0 for elution
KCl	500 mM
MOPS	50 mM
NaCl	1.0 M
NP-40	1%
SDS	1% (with caution)
Tris	50 mM
Urea	8 M

Incompatible reagents

- DTT (dithiothreitol)
- DTE (dithioerythritol)
- EDTA (ethylenediaminetetraacetic acid)
- EGTA (ethylene glycol-bis [β-amino-ethyl ether])

Purification from Crude Cell Lysates

TALON CellThru is a novel IMAC resin for purifying polyhistidine-tagged proteins from crude cell lysates, sonicates, and fermentation liquids. The large bead size of TALON CellThru (300–500 µm) permits cellular debris to flow through the column, eliminating the need for high-speed centrifugation. Additionally, destabilizing factors are removed more quickly with TALON CellThru than with other resins, because the number of steps is reduced.

Advantages of direct capture

Traditionally, obtaining protein from crude cell lysates, such as cell culture and fermentation harvests, requires two steps: isolation, followed by column or batch purification. In the isolation step, the removal of particulate material by centrifugation and/or microfiltration is followed by an initial volume reduction step (typically ultrafiltration). Since conventional chromatography columns are quickly clogged by particles such as cells, cell debris, precipitated proteins, the lysate must be particle-free prior to purification. Therefore, the load must be cleaned before applying it to the column.

However, these centrifugation and filtration steps can be time-consuming and expensive and can also compromise quality. Proteases and glycosidases released from the lysed cells can degrade the target protein, complicate purification, and increase purification costs. The longer the target protein is in the presence of the cell lysate, the more likely it is to be degraded.

One alternative to centrifugation and filtration before loading is a technique called direct capture. With direct capture, you can minimize protein degradation, improve product quality and yield, and save time and money. Also, the initial recovery procedure can be simplified if protein capture and debris removal are combined into a single operation. TALON CellThru allows you to purify His-tagged protein directly from crude cell lysates, including serum, tissue extracts, cell culture harvests, fermentation broth and other crude samples on resin-packed, standard low-pressure columns.

A large agarose bead adsorbent is packed into standard chromatography columns whose end-plate frits (filters) have large pores (190 µm) to prevent column blockage. Because of the large bead sizes, particulate material flows between the beads while the soluble product binds to the immobilized metal ions on TALON Resin. Residual particulate material can be removed from the column by using bidirectional high-speed wash pulses. The product is the eluted by normal elution methods.

Expanded bed chromatography vs. top-loading

TALON CellThru can be used in expanded bed chromatography. With this type of chromatography, the crude lysate is applied to the column in an upward rather than downward direction, resulting in increased distance between resin particles (Anspach, *et al.*, 1999). Using the upward flow, the bed does not become clogged and a greater amount of protein is recovered.

Expanded bed chromatography integrates solid-liquid separation, volume reduction, and partial purification all into one step. The amount of cellular debris can be reduced up to five orders of magnitude. The combination of increased distance between particles and the large bead size of TALON CellThru allows for excellent protein adsorption without clogging the bed.

Purify Protein from Crude Cell Lysates...*cont.*

The yield from a particular expanded bed or CellThru application depends to a large extent on the efficiency of the extraction procedure in promoting interaction of the target proteins with the resin beads. Incomplete lysis will result in perceived losses of the target protein in the cell debris, which is removed by centrifugation.

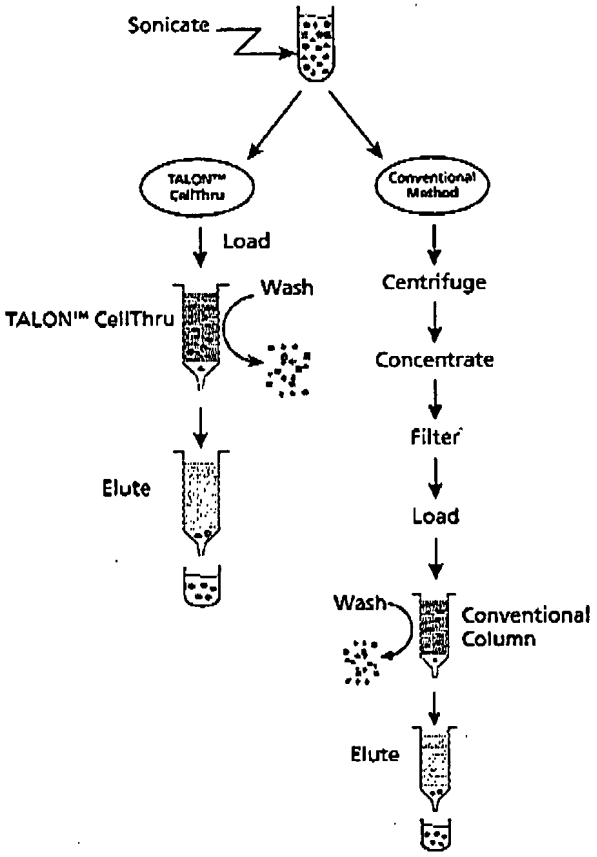

Figure 14. TALON™ CellThru purifies protein from crude cell lysate faster than conventional methods.

Purification from Crude Cell Lysates...*cont.*

CellThru purifies membrane bound proteins and multiprotein complexes

Some proteins are not as easy to access as soluble cytosolic proteins. For example, some recombinant proteins may interact with proteins embedded in the cell membrane (membrane-bound or membrane-associated), while others may be compartmentalized within subcellular organelles. When performing SDS-PAGE analysis, this is generally not apparent because the high SDS and salt concentrations in the sample buffer help solubilize the membranes. Thus, nearly all the proteins present in a cell lysate can be visualized when run on an SDS-PAGE gel.

Purifying membrane-associated proteins with standard TALON Resin is challenging because lysates must be clarified before application to the column. This centrifugation step will usually remove most of the membrane-associated proteins along with the cell membranes and subcellular organelles.

In contrast, with TALON CellThru Resin you can run the crude lysate on the column without centrifuging (direct capture). In this procedure all membranes and unbroken subcellular compartments pass through the column increasing the likelihood of capturing membrane-associated proteins. Therefore, when purifying multiprotein complexes or membrane-associated proteins, TALON CellThru Resin will provide better yields than conventional TALON. However, if a recombinant protein strongly interacts with the membrane or is contained within unbroken subcellular compartments, some proportion of the protein will not be adsorbed by TALON CellThru and will pass through in the wash fractions.

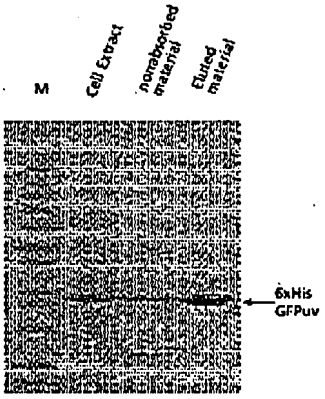

Figure 15. SDS-PAGE of TALON™ CellThru™ purified proteins. *E. coli* BL21 cells were sonicated in TALON wash buffer and run through a TALON CellThru column eluted in 150 mM imidazole. Note that some target protein is trapped in membrane fractions and does not get absorbed on the column. M=molecular weight standards.

TALON Product List	Size	Cat. #
TALON Metal Affinity Resin	10 ml	8901-1
	25 ml	8901-2
	100 ml	8901-3
	250 ml	8901-4
TALONspin Columns	10 cols.	8902-1
	25 cols.	8902-2
	50 cols.	8902-3
	100 cols.	8902-4
TALON 2-ml Disposable Gravity Column	50 cols.	8903-1
TALON Superflow Metal Affinity Resin	25 ml	8908-1
	100 ml	8908-2
TALON CellThru	10 ml	8910-1
	100 ml	8910-2
CellThru 2-ml Disposable Columns	50 columns	8914-1
CellThru 10-ml Disposable Columns	20 columns	8915-1
TALON Buffer Kit	each	K1252-1
TALON Purification Kit	each	K1253-1
Talon-Dextran Trial Size	5 mg	8918-y

Thiophilic Resin Product List

Thiophilic-Uniflow Resin	10 ml	8913-1
	100 ml	8913-2
Thiophilic-Superflow Resin	10 ml	8917-1
	100 ml	8917-2

Glutathione Resin Product List

Glutathione-Superflow Resin	10 ml	8911-1
	100 ml	8911-2
Glutathione-Uniflow Resin	10 ml	8912-1
	100 ml	8912-2
GST Purification Kit	5 purifications	K1251-1

Appendix B

Crop Science 43:1068-1071 (2003)

CELL BIOLOGY & MOLECULAR GENETICS

Mapping of QTL for Embryo Size in Rice

Yanjun Dong*, Eiji Tsuzuki, Hiroshi Kamiunten, Hiroyuki Terao and Dongzhi Lin

Agricultural Faculty, Miyazaki University, Miyazaki City, 889-2192, Japan

* Corresponding author (a01120u@cc.miyazaki-u.ac.jp)

ABSTRACT

The development of molecular genetic maps has accelerated the identification and mapping of genomic regions controlling quantitative trait loci (QTL) in rice (*Oryza sativa* L.). Minimizing embryo size in rice would increase proportion of edible endosperm. This study was conducted to determine the genetic basis controlling embryo size of rice grains in a recombinant inbred (RI) population derived from cross of a japonica cultivar, Asominori, with an indica cultivar, IR24, by means of 289 restriction fragment length polymorphism (RFLP) markers. Two parameters, embryo length and embryo width, which represent embryo size in rice, were estimated for each RI line and their parental varieties. Continuous distributions and transgressive segregations of embryo length and embryo width in rice were observed in the RI population, suggesting that embryo size was quantitative in grains of conventional varieties. Three QTL for embryo length were detected on chromosomes 1, 2, and 3 and explained 17.9, 25.7, and 9.2%, respectively, of the total phenotypic variation. Three QTL for the embryo width were observed on chromosome 2, 8, and 10 and accounted for 13.5, 15.7, and 15.0% of total phenotypic variation, respectively. In addition, alleles with increasing and decreasing effects were detected from the both parents. The results and the tightly linked molecular markers that flank the QTL will be useful in breeding for embryo improvement in rice.

Abbreviations: CIM, composite interval mapping • cM, centimorgan • QTL, quantitative trait locus • RFLP, restriction fragment length polymorphism • RI, recombination inbred

INTRODUCTION

Rice is a staple food for more than 50% of the world's population. The grain consists of endosperm and embryo. The endosperm is the major edible part for humankind. In contrast, the embryo, which is easily broken during processing, is usually used for an industrial material. At present, in rice-breeding programs, relatively little attention has been focused on embryo size. However, during the past two decades, breeding efforts to improve nutritional quality in rice has focused on enlarging the embryo for industrial purposes. Relative to other parts of the rice grain, the embryo has high concentrations of protein, oil, and vitamins (Juliano, 1985; Koh et al., 1994).

Satoh and Omura (1981) first found a giant-embryo mutant from a Japanese cultivar, Kinmaze, and mutant gene was located on chromosome 7 (Satoh and Iwata, 1990). In addition, Kim et al. (1991)(1992) detected three giant-embryo

mutants (ge^m, ge, ge^s), all at the same locus reported by Satoh and Iwata (1990). More recently, Koh et al. (1996) precisely mapped the ge^s locus controlling super-giant embryo to chromosome 7 using RFLP and microsatellite markers.

If the objectives of breeding programs are to increase embryo size, it is clearly important to study the giant-embryo mutants in rice. On the other hand, if the ultimate goal of a breeding program is to increase endosperm components, it is valuable to minimize the embryo size. Thus, an understanding the genetic basis underlying the inheritance of embryo size in rice has significant implications for quality improvement.

The recent advances in high-density marker linkage maps in rice have provided powerful tools for elucidating the genetic basis of quantitatively inherited traits (Causse et al., 1994; Harushima et al., 1998). As a result, numerous QTL (Yano and Sasaki, 1997) associated with yield and its components and other agronomic traits in rice have been identified and mapped by means of molecular markers. However, to our knowledge, genetic analysis of QTL associated with embryo size has not been conducted in normal grain rice. The aims of this study are to identify QTL for embryo size by means of RI lines from a japonica/indica cross and to determine the relationships between QTL for embryo size and QTL for grain size, which were found in previous studies (Redona and Mackill, 1998; Tan et al., 2000).

MATERIALS AND METHODS

▲ TOP
▲ ABSTRACT
▲ INTRODUCTION
• MATERIALS AND METHODS
▼ RESULTS
▼ DISCUSSION
▼ REFERENCES

Plant Materials

The RI lines in this study, kindly provided by professor A. Yoshimura of Agricultural faculty of Kyushu University, Japan, were developed by single seed descent from the progeny of a cross of japonica cultivar, Asominori, with indica cultivar, IR24. 165 F_6 lines were randomly selected from 227 original F_2 individual plants and used for mapping. The RFLP map covering 1275 centimorgans (cM) was constructed with 375 markers from the F_6 and F_7 generations of 71 RI lines (Tsunematsu et al., 1996). In the past, the RI population was used successfully for mapping QTL for important agronomic traits (Yoshimura et al., 1998; Yamazaki et al., 1999; Yamazaki et al., 2000; Sasahara et al., 1999). In this study, we used a subset of 289 RFLP markers, without overlapping, for all loci from the original genetic map (Tsunematsu et al., 1996) to map QTL affecting embryo size in rice, for which the average interval distance between pairs of markers was 4.4 cM.

Measurements of Length and Width of Embryo in Brown Rice Grains

The RI population along with its parents, Asominori and IR24, were grown at the Experimental Station of Miyazaki University, Japan, during summer–autumn 2001 with two replications in accordance with conventional methods. At maturity, rice grains from each line were harvested and dried naturally in the glasshouse. Then 50 grains from each line were randomly selected and hulled by hand with care and embryo length and width of was measured with a microscope under a magnification of 50x. All measurements for each line were replicated three times. Average values for each line were used for statistical analyses.

Detection of QTL

Two methods were used simultaneously to identify significant marker locus-trait associations: simple linear regression (single marker analysis) and composite interval mapping (CIM) analysis. The CIM analysis was applied to trait average and marker data to identify more precisely the QTL locations (Zeng, 1994). Single marker analysis and CIM analysis were performed by QTL Cartographer computer program software (Wang et al., 1999) version 1.13 g. The linkages between respective marker loci and putative QTL were determined by single marker analysis. When F values exceeded a

value necessary for a probability value less than 0.005, the QTL were considered to be significant. CIM analyses were calculated by forward regression, the walk speed of 2 cM, and the window size of 10 cM. A locus with a LOD threshold value of more than 2.5 was to be declared a putative QTL.

In this study, only the QTL detected by both methods were listed. In addition, the additive effect and percentage of variation explained by an individual QTL were also estimated. The QTL were named according to the suggestions of McCouch et al. (1997).

RESULTS

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Distribution of Length and Width in Embryo in Segregating RI Population

The average length and width for embryo of both parents (Asominori, IR24) and the frequency distributions of RI lines are presented in Fig. 1. The two parents, show differences in embryo size, especially for embryo length. Continuous phenotypic variation of both embryo length and width and transgressive segregation suggested that embryo length and width were quantitative traits.

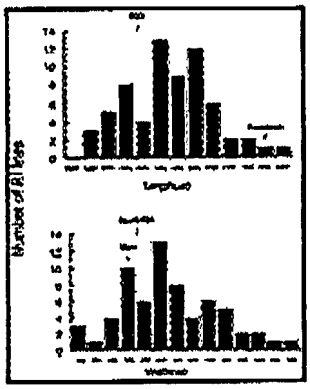

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Fig. 1. Frequency distributions for length and width of embryo in rice grains using RI lines derived from a cross between Asominori and IR24.

Mapping QTL for Embryo Size

Embryo Length

Three QTL controlling the embryo length were identified and mapped to chromosomes 1, 2, and 3 (Table 1 and Fig. 2) and tentatively named for *q*EML-1, *q*EML-2, and *q*EML-3, respectively. The *q*EML-2, located near C132 markers on chromosome 2, showed the largest effect on the trait with a LOD value of 4.3 and explained 25.7% of the total phenotype variation. Another QTL, *q*EML-1 (LOD = 3.7), was detected near XNpb393 on chromosome 1 and accounted for 17.9% of total variation. The remaining QTL, *q*EML-3, located near R1468B on chromosome 3, with a LOD value of 2.8, explained 9.2% of total phenotype variation. In addition, the IR24 alleles in both *q*EML-2 and *q*EML-3 contributed to the decrease in embryo length, whereas *q*EML-1 locus increased the embryo length.

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Table 1. QTL controlling embryo size in rice based on composite interval mapping (CIM) and single marker analysis methods (Wang et al., 1999) using RI lines derived from a cross between Asominori and IR24.

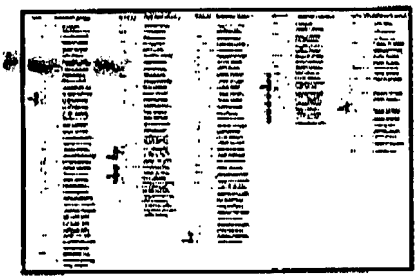

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Fig. 2. Chromosomal locations of QTL for embryo size in RI population derived from the cross between Asominori and IR24. Black and shaded bars indicate the genomic regions with $P < 0.01$ of QTL detected for embryo length and embryo width, respectively, based on single marker analysis. Black arrowheads indicate the location of peak LOD for QTL detected.

Embryo Width

Three QTL (Table 1 and Fig. 2) affecting the embryo width were detected and mapped to chromosomes 2, 8, and 10, and tentatively designated as *q*EMW-2, *q*EMW-8, and *q*EMW-10, respectively. *q*EMW-8 had the largest effect (LOD = 5.1) and was located near XNpb41 on chromosome 8 and explained 15.7% of total phenotype variation. *q*EMW-2 had a LOD value of 2.6, was located near R712 on chromosome 2, and explained 13.5% of total variation; and *q*EMW-10 was located near C1361 marker on chromosome 10 and accounted for 15.0% of total phenotype variation. Further, the two alleles from the indica parent, IR24, in *q*EMW-8 and *q*EMW-10 decreased the embryo width, while *q*EMW-2 allele increased the embryo width.

DISCUSSION

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In this study, we report the results of QTL mapping for embryo size with 289 RFLP markers in conventional cultivars using the RI lines derived from japonica Asominori and indica IR24. In the past, the set of RI lines used in our study was used to analyze QTL for days to heading (Yoshimura et al., 1998), whitebacked planthopper (*Sogatella furcifera* Horvath) (Yamazaki et al., 1999), ovicidal response to brown planthopper (*Nilaparvata lugen* Stal) (Yamazaki et al., 2000), and vascular bundle system and spike morphology (Sasahara et al., 1999). From those results, it was shown that these RI lines are very useful for identification of QTL in rice.

Three QTL (*q*EML-1, *q*EML-2, *q*EML-3), located on chromosomes 1, 2, and 3, for embryo length and three QTL (*q*EMW-2, *q*EMW-8, *q*EMW-10), located on chromosome 2, 8, 10, for embryo width were detected. In addition, alleles with increasing and decreasing effects for embryo size were detected from the both parents. The indica parent, IR24, had decreasing alleles for embryo size at *q*EML-2, *q*EML-3, and *q*EMW-2, but increasing alleles at *q*EML-1, *q*EMW-8, and *q*EMW-10, while Asominori alleles had the opposite effects. These results could explain the transgression and continuous distributions for embryo size in the RI population. It is noted that all six QTL detected in the study were independent of the three alleles (ge^m, *ge*, ge^s) that control the giant-embryo trait in rice, located on chromosome 7 in

previous studies (Kim et al., 1991; Satoh and Iwata, 1990; Koh et al., 1996).

However, in an attempt to increase the utilization of rice grains by minimizing embryo dimensions in normal grains, it is a prerequisite that there be neither tight linkages nor pleiotropic effects between QTL for embryo size and grain size. In the past, Tan et al. (2000) reported three QTL for grain length, located on chromosomes 3, 6, and 7 and three QTL for grain width on chromosomes 1, 5, and 8, respectively. Redona and Mackill (1998) also identified seven QTL for grain length, located on chromosomes 2, 3 (two regions), 4 (two regions), 7, 10, and four QTL for grain width, located on chromosomes 2, 3, 7, 8, using an F_2 population derived from tropical japonica/indica cross. In comparing the genomic positions of the QTL identified by Tan et al. (2000) and Redona and Mackill (1998) for grain size with the six QTL detected in our studies for embryo size, *q*EMW-8 on chromosome 8 is tightly linked to or allelic to the one for grain width reported by Tan et al. (2000) and Redona and Mackill (1998); furthermore, *q*EML-3 on chromosome 3 might be closely linked to a QTL for grain length (Redona and Mackill, 1998), which was obviously different from the one QTL reported by Tan et al. (2000). The other four QTL (*q*EML-1, *q*EML-2, *q*EMW-2, and *q*EMW-10) for embryo size discovered in this research were different from grain-size QTL located in other genomic regions. Hence, these four QTL could be used for rice embryo improvement since they do not affect whole grain size.

In summary, the results from this study can be useful for increasing the utilization of edible endosperms of rice grains by decreasing embryo size in conventional cultivars. The closely linked molecular markers that flank four QTL (*q*EML-1, *q*EML-2, *q*EMW-2, *q*EMW-10) that are independent of grain-size QTL detected in our studies should be very useful for marker-assisted breeding to improve embryo size in rice when transferring the quantitative genes in breeding programs.

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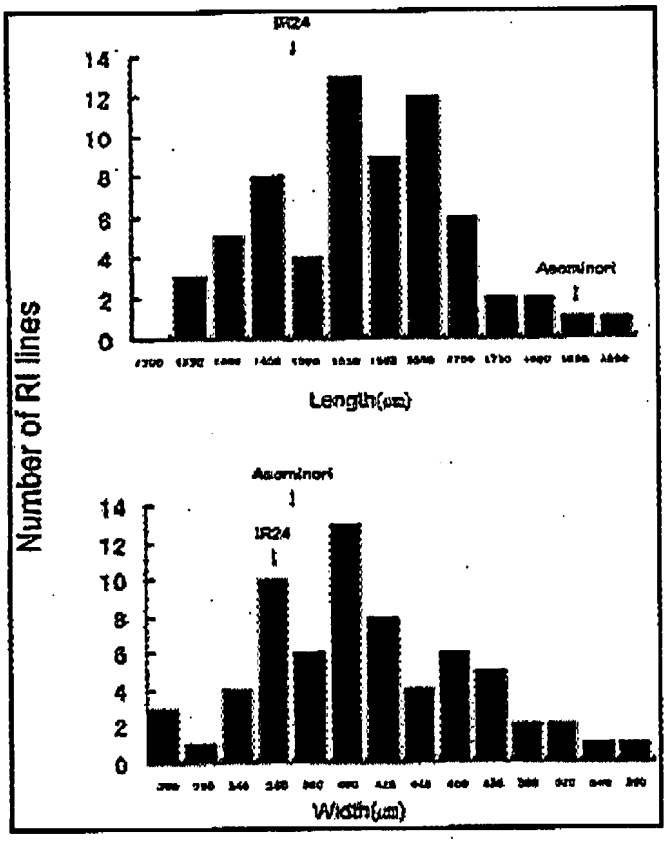

Fig. 1. Frequency distributions for length and width of embryo in rice grains using RI lines derived from a cross between Asominori and IR24.

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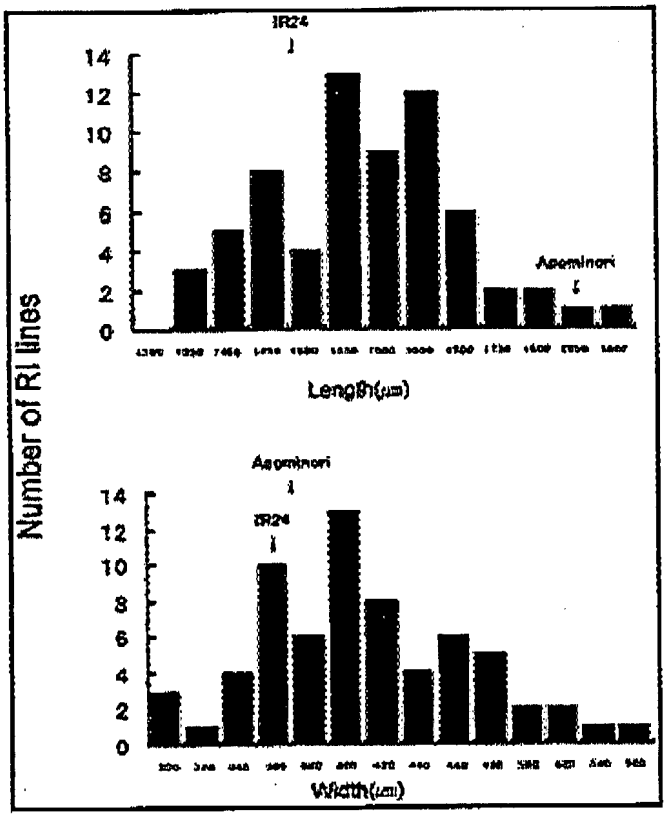

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